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A Flow-Through Testing Procedure with Duckweed (Lemna minor L.)

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A FLOW-THROUGH TESTING PROCEDURE WITH DUCKWEED (*LEMNA MINOR* L.)

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FOREWORD

Our nation's fresh waters are vital for all animals and plants, yet our diverse uses of water -- for recreation, food, energy, transportation, and industry -- physically and chemically alter lakes, rivers, and streams. Such alterations threaten terrestrial organisms, as well as those living in water. The Environmental Research Laboratory in Duluth, Minnesota, develops methods, conducts laboratory and field studies, and extrapolates research findings

--to determine how physical and chemical pollution affects aquatic life,

--to assess the effects of ecosystems on pollutants,

--to predict effects of pollutants on large lakes through use of models, and

--to measure bioaccumulation of pollutants in aquatic organisms that are consumed by other animals, including man.

This report describes a flow-through testing procedure for duckweed. It identifies the variables that have to be controlled and the ranges to which they should be confined. Copper sulfate is used as an example toxicant.

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ABSTRACT

Lemna minor is one of the smallest flowering plants. Because of its floating habit, ease of culture, and small size it is well adapted for laboratory investigations. Procedures for flow-through tests were developed. Testing procedures were developed with this apparatus. By using the techniques described here, the effects of nutrients or toxicants, singly or in combination, can be determined in several concentrations with several replicates of each. Responses which can be measured include changes in growth rate, changes in death rate, changes in timing of division of colonies, color changes, changes in variability, and modification of the flowering response. Emphasis here is on changes in growth rate determined either by daily frond counts or by final frond numbers.

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SECTION I

INTRODUCTION

Lemna minor L., lesser duckweed, is common throughout the world. In fact, it is absent only from the polar regions and a few parts of the tropics. Lemna minor is a small floating plant consisting of a single leaf-like frond that is flat, more or less oval in outline, and 1.5 - 4 mm long. A single root is attached about a third of the way from the narrow end of the frond. The root, which is terminated by a prominent calyptra, or root cap, may be as long as 10 cm. Vegetative reproduction is accomplished by the growth of daughter fronds alternately from a pair of reproductive pouches at the sides of the root-bearing end of the frond. Flowering is rare, but the flowers also arise from the reproductive pockets. A Lemna colony is made up of several generations of daughter fronds still attached to the parent. In its natural environment L. minor is eaten heavily by some wildfowl, beaver, and muskrat. It serves as physical support for epiphytic organisms, which are in turn eaten by aquatic crustaceans, molluscs, annelids, and insect larvae. For fish L. minor provides food, shelter, and shade, though it tends to shade out other aquatic plants that might produce food more efficiently (Muenscher 1944, Sculthorpe 1957).

The Lemnaceae in general have a number of characteristics that make them useful laboratory organisms, such as ease in obtaining clonal material, simple methods of axenic culture, and small size. A consequence of this combination of properties is that a large background of research has been developed for this species.

Static tests are conducted in a non-renewed solution of the material under investigation. Also, the plants may be transferred into fresh solutions during the test. Several authors describe applicable techniques. Fromm (1946, 1951, 1960) used greenhouse lighting and 20 plants per flask with four flasks per concentration. Mitchell et al. (1958) recommended sterilization of the plants and culture medium for some uses. Further, they control light (700 - 1000 ft-C for 12 hrs per day) and temperature (25°- 27°C). Blackman (1952) controlled light and temperature. He did not use sterile solutions or sterilize the plants, but instead changed solutions every 48 hrs to keep contamination down as well as to keep the pH and the chemical concentrations within reasonable ranges. Fekete et al. (1976) used a bioassay procedure to evaluate the release of available phosphorus from pond sediments. Here again, greenhouse lighting was used.

Advantages of the static and renewal procedures lie in the small quantities of solution required and in the simplicity of the experimental design; no delivery system is required. The major limitation of a static experiment is

that the concentrations of active materials in the experimental container may change over the test interval through degradation, precipitation, etc.

Flow-through and recirculating systems of varying degrees of sophistication have been designed. These systems range from single open chambers, through which the solution moves by a siphon arrangement (Mendiola 1919), to complex aseptic systems in which the area of a raft of plants can be kept constant so that the effects of crowding can be eliminated (Erismann and Pinger 1968) or systems in which samples may be taken automatically without violating aspects (Strasser 1971).

Ashby et al. (1928) designed a recirculating system that was not aseptic. Eichorn and Augsten (1969) cultured Wolffia arrhiza in a chemostat under continuous illumination.

The Lemnaceae have also been used in systems for testing the effects of various air pollutants on plants. Todd et al. (1956) tested the effects of air containing ozone and ozonized hexane. Feder and Sullivan (1969) studied the reduction of frond multiplication and floral production by ozone. Schürer et al. (1975) showed that Lemna minor could metabolize sulfur dioxide from the air if it was made available in sublethal concentrations.

The cultivation and testing of Lemna minor have produced a number of ways to quantify responses. These depend on such things as frond death, premature division of colonies, alteration of flowering response, and dry weight changes. At least two methods of visually evaluating frond condition, are available. One is to assign degree-of-damage numbers to the fronds, e.g., from zero (no damage) to five (complete loss of green color) (Parker 1965). The second visual method involves frond counts, which have been shown to be a reliable measure of growth response in the Lemnaceae. These counts compare well with dry weight changes and area changes. Ashby and Osley (1935) and Fromm (1960) have used this technique.

The genus Lemna is unusual in that plants are not damaged by what might be considered excessive day lengths, including continuous illumination with no dark period. The continuous growth of Lemna in a 24-h photoperiod is useful in reducing the duration of the tests being performed (Clark 1925, Landolt 1957).

The light intensities that can be used by Lemna minor range from 1,600 to 17,000 lux, but there is no appreciable increase in growth above about 8,000 lux (Ashby and Osley 1935). The lower end of this range is easily attainable in laboratory situations.

The procedures described above were not designed with simplicity in mind, so this work was undertaken to provide a fast, simple, and inexpensive technique for evaluating stimulatory or inhibitory effects of pollutants on a typical aquatic plant.

SECTION 7

CONCLUSIONS AND RECOMMENDATIONS

The lesser duckweed (Lemna minor L.) is a suitable higher plant for testing in a flow-through system. The testing method developed in this study should be useful for evaluating effects of toxicants, enrichments, and otherwise altered conditions on higher aquatic plants. A procedure for using Lemna minor as a test organism is recommended.

SECTION 3

METHODS

Physical Methods

Lake Superior water, temperature-controlled but otherwise unmodified, was delivered at regular intervals by use of a delivery chamber that periodically emptied itself by a self-starting siphon (Hero of Alexandria, ca. 62 AD). The periodic flow of water operated a chemical-metering apparatus designed for highly water-soluble materials (Mount and Brungs 1967) (Figure 1). This apparatus added a solution of plant nutrients to the feedwater of a proportional diluter (Mount and Brungs 1967). This diluter was provided with a mechanical syringe injector which added copper sulfate when this chemical was used as a toxicant (DeFoe 1975). The diluter delivered the resulting series of concentrations through glass delivery tubes into battery jars. The jars had a capacity of 1.4 l each. The delivery tubes were wrapped in black polyethylene film to reduce growth of attached algae on the inner surfaces. Toxicant was added by an injector system designed to add microliter quantities to the water to operate as the diluter cycled.

The battery jars drained through a notch cut 6 cm down from the top edge. A silicone-based glass and ceramic adhesive was used to fasten stainless steel boiling cloth (screen) across this opening. This allowed the water to drain out, but prevented any loss of experimental plants. The screen used here was 105 mesh with 0.165-mm openings.

Four separate battery jars were used for each concentration of the material being tested. Individual colonies of duckweed were kept separated and centered in the jars by seven-compartment floats (Figure 2). The separator consisted of seven rings, 1-cm sections of 2-cm glass tubing; six of these were glued in a hexagon around the seventh. This separator was glued between and supported by two air-filled 4-, dram glass vials with plastic caps. The float was kept centered with a hook made of bent stainless steel wire which was hung over the edge of the jar. The separation of the colonies made it possible to monitor each one individually. An index mark on one float showed the starting point so that data for each colony could be recorded separately and consistently (Figure 2).

Continuous illumination was provided by fluorescent tubes, half of which were Duratest Optima and the remainder Gro Lux, Wide Spectrum. The distance from the fluorescent tubes to the frond level was 20-25 cm. This provided a light intensity of 3,700 lux; the standard deviation (s) was 2,50 lux.

Temperatures in the test chambers were maintained at 22.8°C (s = 0.6°). Alkalinity, pH, hardness, and acidity were measured at the beginning and the end of each experiment (American Public Health Association 1971).

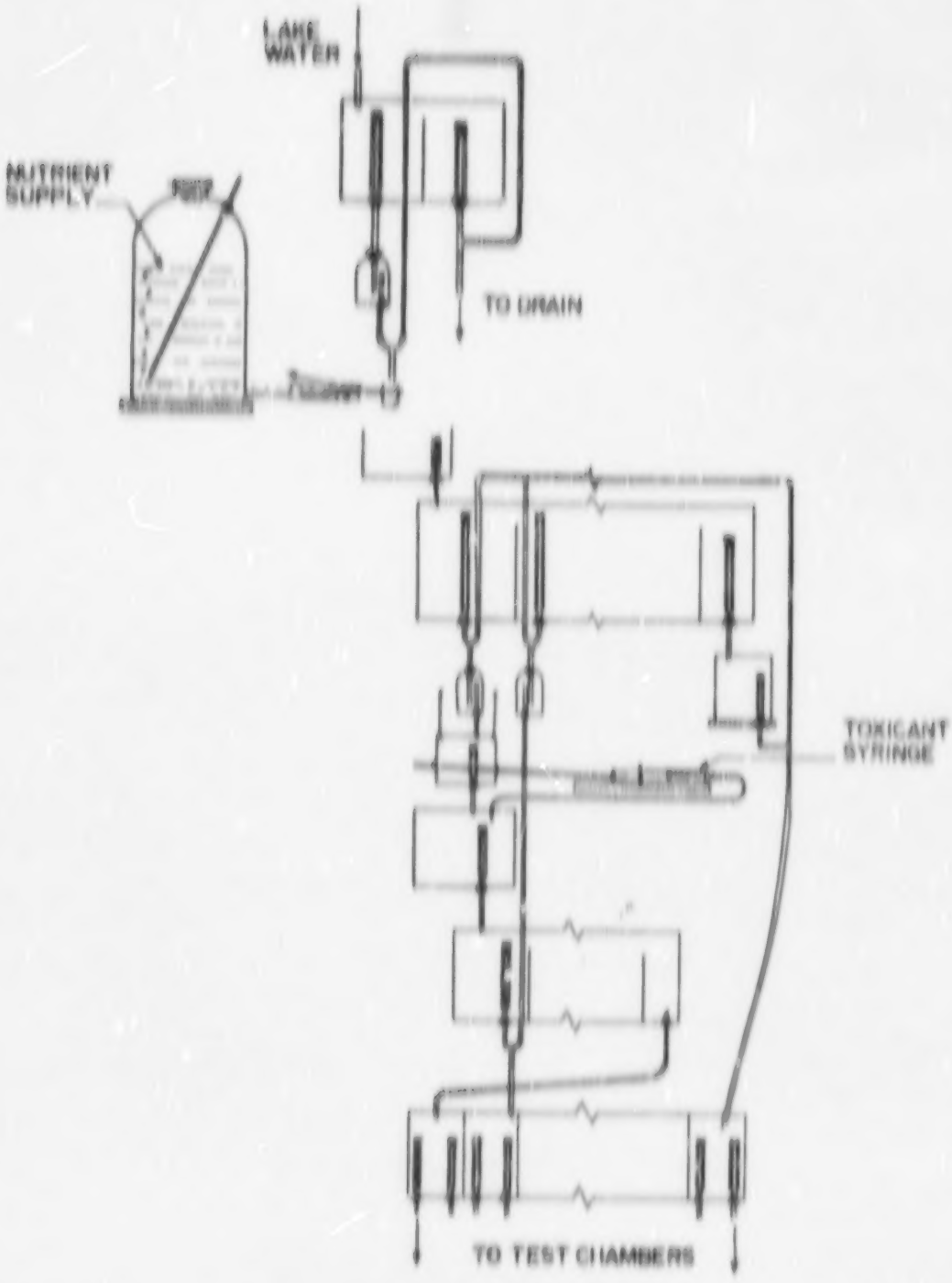
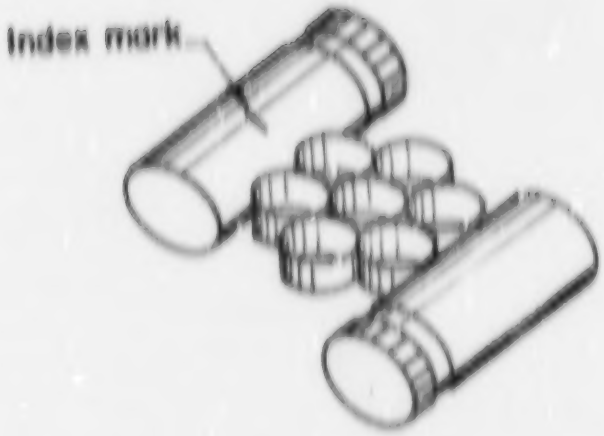
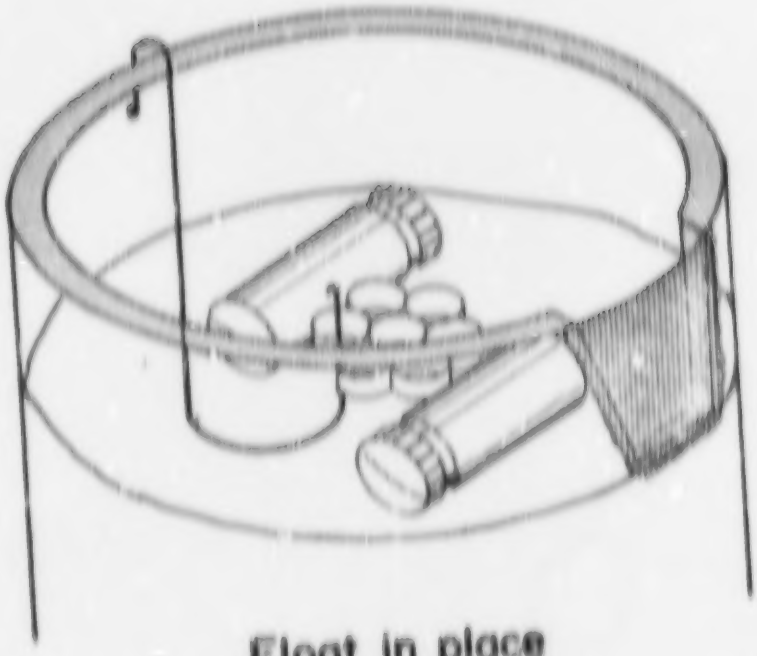


Figure 1. Nutrient- and toxicant-adding diluter.



Float



Float in place

Figure 2. Duckweed rooting chamber and float.

The flow rate into the system was 1.2 l of water (s = 0.13 l) delivered every 15.6 min (s = 2.0); this was mixed with 2.5 ml (s = 0.15 ml) of nutrient solution.

Aeration of the test chambers was not necessary. Lemna minor fronds have adequate gas exchange through their dorsal surfaces.

Biological Methods

The clone that was used (Lemna minor L. #6) was originally collected in Florida in the vicinity of Boca Raton some time before 1964. This clone has been in culture at the Environmental Research Laboratory-Duluth since 1971. Except where otherwise noted, colonies of two fronds each were used to start a test. One colony was placed in each retaining ring of the float. This resulted in seven colonies per jar and, since each concentration was replicated four times, 28 colonies per test concentration.

To reduce possible effects of attached algal growth on surfaces in contact with the test solution, on the 4th day of a 7-day test the colonies were transferred into clean floats in clean battery jars. The water in the replacement battery jars was lake water that had been allowed to equilibrate to room temperature for 16-24 hrs. When copper toxicant was used, the water in the jars was dosed with the appropriate concentrations of copper before the transfer. Thus the duckweed was continuously exposed to the copper concentrations. In the copper experiment the glassware cleaning procedure included acid washing.

In the course of experimentation two kinds of nutrients were used. The first was a commercial soluble fertilizer (Garden Life, Science Products Company, Chicago, Illinois 60614) with an analysis 16% nitrogen, 15% phosphorus, and 14% potassium. The stock concentration was 1.2% mg/l. The second nutrient was Hutner's Medium (Hutner 1953).

Stock cultures were maintained continuously under the same conditions as the test organisms, lacking only the toxicant additions.

Growth rate (K) was calculated by the formula

$$K = \frac{\text{Log}_{10}\ (Fd) - \text{Log}_{10}\ (Fo)}{d}$$

where Fo is the original frond number, Fd is the frond number on day d, and d is the number of days involved (Hillman 1961). When analysis of variance or regression were used, individual frond counts were used, not chamber averages.

Plant response for the first 2 or 3 days reflects a combination of experimental and pre-experimental conditions; therefore, growth rates from this period were not used (Fromm 1960). Because it is difficult to maintain identical light intensities at the surface of the experimental containers

and because the light output of fluorescent tubes changes with time, two experiments were undertaken to determine whether the light variations over the test bench correlated significantly with frond numbers at termination.

An experiment was undertaken to correlate the effects of the differing flow rates into the separate containers with the frond numbers at termination. A comparison was made between a series of static concentrations of a commercial soluble fertilizer (Garden Life) and a continuously replaced solution of the same material.

The purpose of another experiment was to determine when reduction of growth due to crowding within the individual float chambers became significant. Chambers were given six two-frond colonies each instead of one.

Because unequal amounts of damage may be caused in handling or in the reduction of the colonies to two-frond units, minimally handled colonies were compared with colonies that might be expected to show a maximum response to handling. The unmodified colonies were simply transferred intact from stock cultures to test chambers. The modified colonies were carefully broken into two-frond units and the roots were excised.

The method developed here was tested with copper sulfate as the toxicant. ED50 used here indicates a reduction of the production of new fronds by half; this is derived from the difference between the final frond number and the original frond number. Because copper at 31 ppb produced more growth than the control Lake Superior water at 1 ppb, growth reduction was calculated from this level as a starting point.

SECTION 6

RESULTS

Effect of Light Variation

In two experiments no correlation was found between variations in light intensity at frond level and frond numbers at termination. The mean of the first experiment was 2,317 lux (s = 219) and of the second experiment, 2,749 lux (s = 221).

Effect of Flow-Rate Variation

A significant correlation (0.57) was found between flow rate and final frond number in each test chamber (Figure 3).

Effect of Static vs. Flowing Conditions

The flowing concentrations of the soluble fertilizer at 1.24 mg/l produced growth that was not significantly different from the static concentration of the same fertilizer at 100 mg/l (Figure 4).

Effect of Handling

Reduction of the colonies to two-frond, root-excised colonies did not produce a significant reduction in growth rate.

Effect of Copper Sulfate

Linear regression was used for the data from 31 ppb to 210 ppb. The 7-day ED50 was 119 ppb copper, the upper confidence limit was 151, and the lower confidence limit was 105 (Figure 5).

Effect of Crowding

A statistically significant reduction in growth was noted when the area within the individual float chamber was reduced to 8.0 mm^2 per frond. Three days before this area was reached the available area was 12.5 mm^2 per frond.

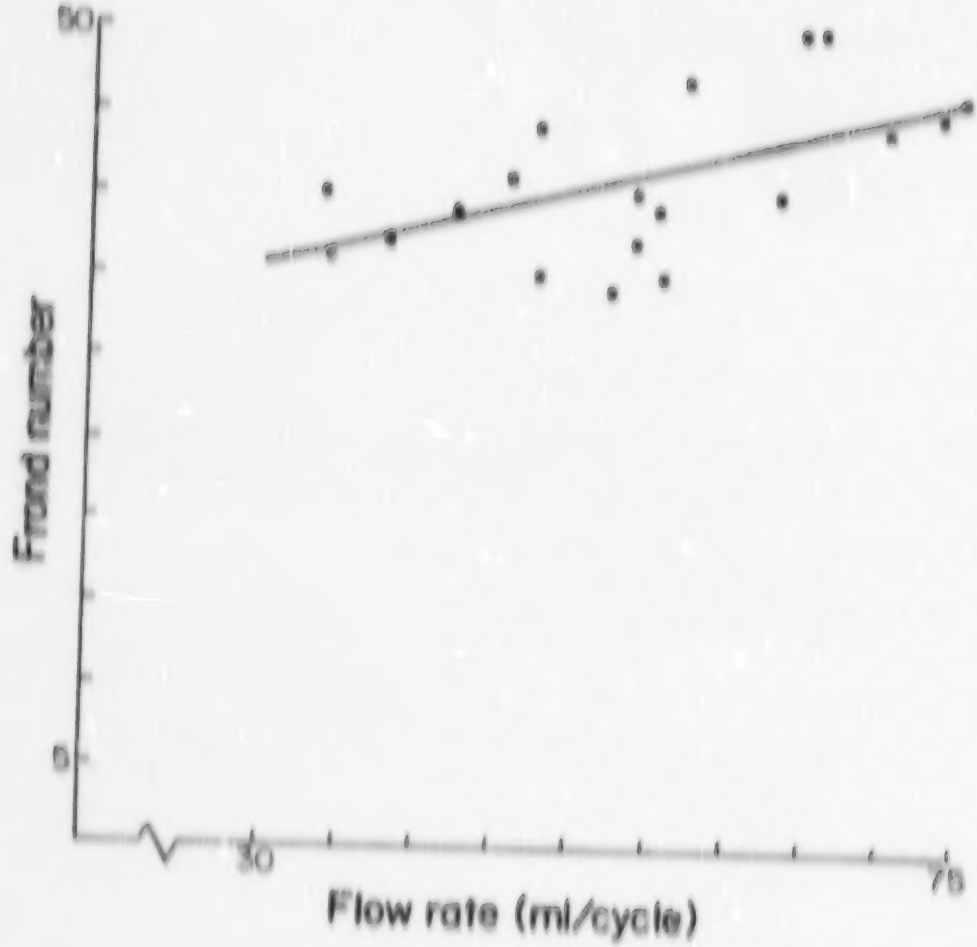


Figure 3. Correlation between flow rate in chambers and frond numbers at end of 7 day test. Cycle time is 3.86 cycles per hour.

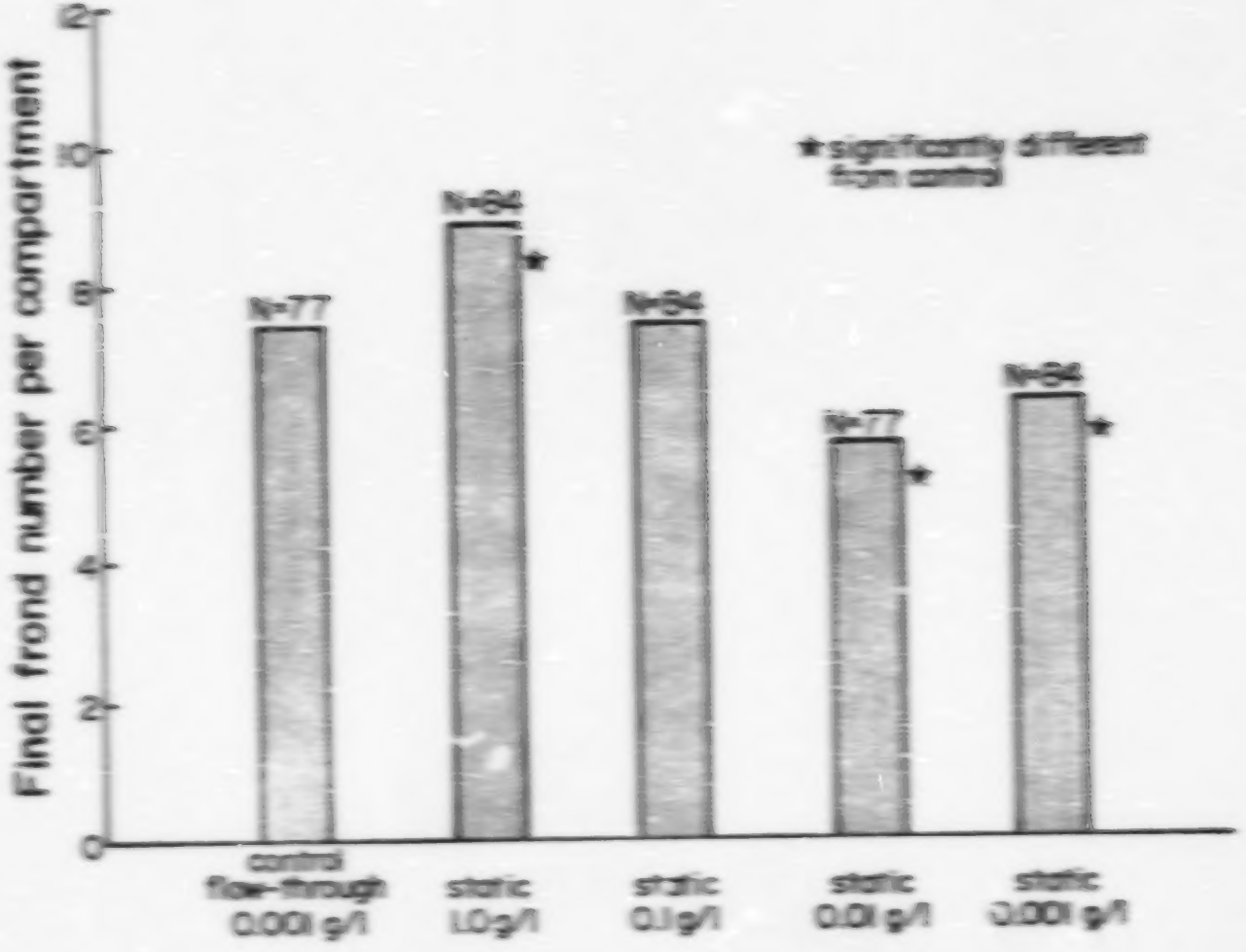


Figure 4. Growth differences between flowing and static conditions at various nutrient concentrations.

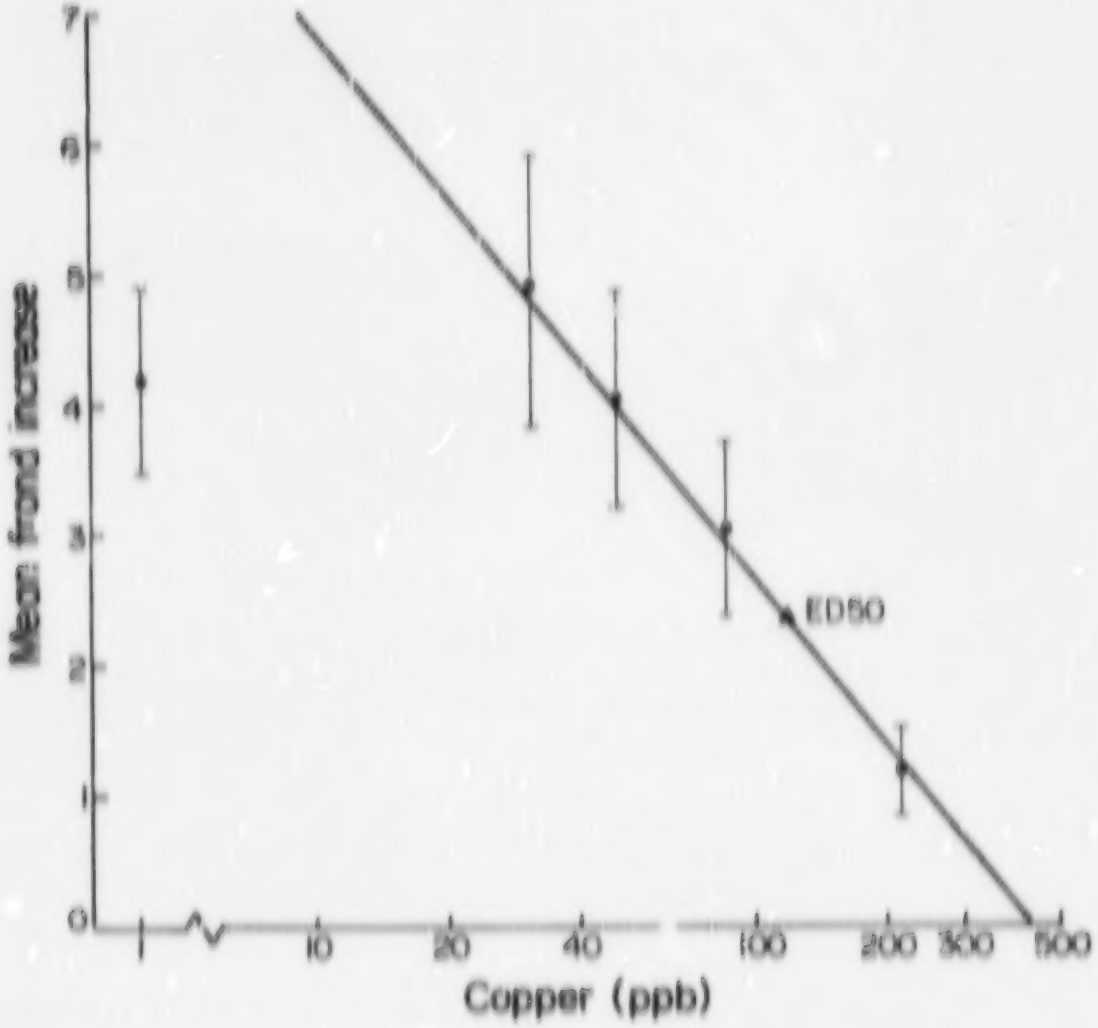


Figure 5. Growth responses to copper, as copper sulfate.

SECTION 5

DISCUSSION

These experiments were undertaken in an attempt to develop a flow-through testing procedure with duckweed as the test organism. The method was produced by combining earlier work in this area with simplifications derived from experiments. The goal was to create fast, simple, and inexpensive techniques for evaluating stimulatory or inhibitory effects of various pollutants. Where comparison is possible, the results produced here are consistent with those reported earlier. However, in much of the research with duckweed one or more of the important variables are poorly controlled.

Light output of the fluorescent tubes can vary over a fairly wide range, both in space (across the table) and time (between experiments), so it becomes necessary to monitor these changes and periodically determine whether there are significant correlations between light intensities and growth rates over the intensity range utilized. The combination of fluorescent tubes used here (50:50 Gro Lux Wide Spectrum and Duro Test Vita Light) is standard practice at the Environmental Research Laboratory-Duluth. The industry-derived Color Rendering Index (CRI) for each of these is 55 and 92, respectively. Daylight is assigned a CRI of 100. If a better approximation of daylight is needed, only tubes having a CRI of greater than 90 should be used.

Flow rates vary among chambers, and growth rates can vary significantly with them. This flow variation should be kept to a minimum, and the correlation between flow rate and growth rate should be determined periodically.

Flowing conditions require far less in the way of nutrient concentration. This can have the effect of reducing the amount of interaction between the necessary nutrients and the material being tested.

The reduction of the colonies to a standardized colony (two fronds, roots excised) causes no reduction in growth rate. It then is reasonable to start with such a standard colony for reasons of uniformity and because the statistical treatments are somewhat simplified.

In the test of copper sulfate toxicity, which served here only as a test of the procedure, it should be noted that the 7-day EC50 of 119 ppb copper indicates only part of the copper toxicity problem with duckweed. Copper is one of several metals that bioaccumulate in plants so that subsequent complications are to be expected.

The effects of crowding were apparent at 8.0 mm^2 per frond, but because the response of duckweed takes an area of 2-3 days to appear, 12.5 mm^2, the area per frond 3 days earlier, is taken as the upper limit of allowable crowding.

This system or a similar one may be adapted to study the effects of air pollution on plants.

RECOMMENDED METHODS

The following recommended experimental conditions and procedures are based on the results of the experiments described.

1) Initial conditions and measurements:

a) Determine whether the variations in growth are correlated with variations in light intensity or flow rate. In any case, light intensity and flow rate should be as constant as possible. Because changes in light intensity and flow rate are relatively slow, measurements may be made between, not during, experiments.

b) For starting the test and replacing the glassware, use water of the same temperature and the same concentration of toxicants as that from which the plants are being transferred.

c) The stock cultures of experimental plants should be maintained in a situation as close to the experimental conditions as possible. A two-frond, root-excised standard colony reduces transfer of epiphytic algae and makes growth rates of colonies directly comparable.

d) A proportional diluter is not necessarily the only way to provide the toxicant solutions, but any system used should have fail-safe operation, so that if water flow stops, toxicant flow stops as well.

2) Steps should be taken to reduce algal growth wherever possible. The experiments should be designed so that individual colonies can be kept track of separately. Lighting should be continuous.

3) Light intensity should be around 2,500 lux with a standard deviation of 250 or less.

4) Temperatures in the test chambers should be held close to some point between 22° and 25°C with a standard deviation of 0.6°C or less. Infra-red radiation from the fluorescent tubes keeps the chambers slightly above room temperature.

5) For most purposes a 7-day exposure is adequate, but the first 3 days of data are not useful because the plants are still adjusting to their new environment. The glassware may be changed to reduce the influence of epiphytic organisms on or about the fourth day of the test.

6) Toxicant concentrations as well as pH, hardness, and alkalinity should be measured at least three times in the course of a 7-day experiment. The most convenient times are when the fronds are added, when the chambers are changed to reduce periphyton growth, and when the experiment is terminated.

7) Aeration of test chambers is not necessary.

8) Fronds should be counted at the same time every day.

9) If a notched battery jar chamber is used as described here, stainless steel screen over the notch is not essential. Any reasonably inert material will do to hold back fronds that might escape their enclosures. Whatever form the chambers take, the table they rest on should be marked so that they can be placed as consistently as possible.

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